The distribution of calcium in bovine spermatozoa and seminal plasma in relation to cold shock

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Summary. The cause of the higher total calcium level in bovine seminal plasma than in spermatozoa is due mainly to a much higher concentration of complexed calcium in seminal plasma than in spermatozoa. The concentration of protein-bound calcium was approximately the same in spermatozoa and seminal plasma; that of ionized calcium lower in seminal plasma than in spermatozoa.

Sudden cooling (cold shock) of fresh bull semen, which irreversibly abolished motility, led to a significant increase in the concentration of total calcium in spermatozoa, and a corresponding decrease of total calcium in the seminal plasma; the effect was more marked when semen was rapidly cooled to 0°C than to 5°C. The cold-shock induced change in the distribution of calcium between spermatozoa and seminal plasma was reflected in a decreased content of ionized calcium in the seminal plasma and a simultaneously increased content of complexed and protein-bound calcium in spermatozoa. No increase of calcium in spermatozoa was observed following slow cooling under conditions when motility had not been lost irreversibly. Heat inactivation by itself had no marked effect on the calcium concentration ratio between spermatozoa and seminal plasma, but cold-shocking of spermatozoa that had first been irreversibly immobilized by heating produced an increase in the level of sperm calcium. The increase in the calcium level occurred irrespective of whether the spermatozoa had been initially motile. When spermatozoa were reversibly immobilized by treatment with formaldehyde at a low concentration, the calcium level remained unchanged, increasing significantly after cold shock, but not to the same level as in the formaldehyde-free control sample. Addition of EDTA or detergents to the semen prevented the accumulation of calcium by cold-shocked spermatozoa.

Introduction

In the semen of man, bull, ram, rabbit, dog and fowl the spermatozoa are distinguished by the presence of a high level of potassium but a low level of sodium, whereas the converse is true for the seminal plasma (Cragle *et al.*, 1958; Quinn *et al.*, 1965). This suggests the existence of an outward sodium pump mechanism in spermatozoa, similar to that postulated for erythrocytes (Glynn, 1956; Rummel *et al.*, 1961). Two divalent cations, namely calcium and magnesium, also show higher concentration in the spermatozoa than in the seminal plasma in the animal species mentioned above, except the bull, in which calcium concentration in seminal plasma is higher than in the spermatozoa (Quinn *et al.*, 1965).

Sudden cooling of semen or so-called cold shock produces substantial changes in the cation concentration within spermatozoa, namely a decrease in potassium and magnesium and a rise in sodium and calcium concentration (Blackshaw & Salisbury, 1957; Quinn & White, 1966). The mechanism of this phenomenon is unknown and it is not clear why spermatozoa of some species are more resistant to cold shock than others.

The purpose of the present study was to examine more closely the mechanism of cold shock,

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with special reference to the effect which cold shock has on the distribution of calcium in spermatozoa and seminal plasma.

Materials and Methods

Spermatozoa and seminal plasma

Bull semen was collected by artificial vagina. When large volumes were required ejaculates were pooled. Semen intended for experiments was kept at 20–25°C and care was taken to prevent sudden changes in temperature. Only samples of good initial motility were used. In order to separate spermatozoa from seminal plasma the semen samples were centrifuged at 4000 *g* for 30 min.

Chemicals

Ethylenediaminetetra-acetic acid (EDTA, lithium salt) was prepared as described by Long & Mouat (1973). Ammonium salt of tetramethylmurexide was synthesized via tetramethylalloxanthine from caffeine (Cope *et al.*, 1941; Gysling & Schwarzenbach, 1949). All other chemicals were commercial preparations of analytical grade. Calcium-free deionized water was used throughout.

Treatment of semen

Cold-shocking was carried out by running semen along the chilled walls of a large-diameter test tube immersed in ice-water (Mann & Lutwak-Mann, 1955); microscopic examination showed that spermatozoa treated in this manner remained immotile when rewarmed. For slow cooling, the test tube with semen was immersed in a large beaker with water at room temperature and the beaker placed in a cold room at 5°C. Under these conditions at least 2 hr were required for the temperature of the semen to fall from room temperature to 5°C. Microscopic examination showed that under such conditions the spermatozoa became motile on rewarming. In addition to cold shock, two other methods were used to render the spermatozoa immotile. One depended on heating the semen at 50°C for 10 min; this method led to an irreversible and almost complete loss of motility. The other method depended on diluting semen 1:1 with Ringer-phosphate medium, pH 7.4 (Brooks & Mann, 1972), to which 0.00625% formaldehyde had been added. This method also caused loss of motility, which could be reversed, however, by removing formaldehyde by dialysis (Dott & Foster, 1975). Homogenization of semen was done by sonication in an Ultrasonic Cell Disruptor K100 (Kerry's Ultrasonics Ltd, Hitchin, Herts) for 15 sec, as described by Harrison (1974). Freezing and thawing of spermatozoa were carried out as follows. Spermatozoa were separated from seminal plasma by centrifugation and the tube containing the sperm pellet was immersed first in liquid nitrogen (–196°C) for 10 min and then thawed in a water bath at 10°C; this procedure was repeated twice and the pellet was next resuspended in fresh seminal plasma. In order to separate ultrafiltrable and non-ultrafiltrable seminal constituents, seminal plasma and the suspensions of spermatozoa in 140 mM-NaCl were both subjected to ultrafiltration at room temperature over a 24-hr period, using the apparatus described by Lavon *et al.* (1972).

Analytical procedures

Contents of total calcium, magnesium, sodium and potassium were determined in aqueous suspensions of spermatozoa (5–15 mg wet wt) and in seminal plasma (10–50 µl) in an atomic absorption spectrophotometer SP 90 (Pye-Unicam Ltd, Cambridge), using an air-acetylene flame. Lanthanum chloride was added to eliminate interference from phosphate. Spermatozoa and seminal plasma were also analysed for ionized (free), complexed and protein-bound calcium. Ionized calcium was determined in the ultrafiltrates according to the two-wavelength spectrophotometric method of Pedersen (1970); the ammonium salt of tetramethylmurexide served as indicator, and the colour shift was measured at 10°C in an Unicam SP 800 recording spectrophotometer, fitted with automatic

cell changer and constant-temperature cell housing. Complexed calcium was defined as the difference between ultrafiltrable and ionized calcium. Protein-bound calcium was defined as the difference between total and ultrafiltrable calcium (without Donnan corrections). Citric acid was determined by the method of Speck *et al.* (1946) as modified by Mann *et al.* (1949). Acid-soluble phosphorus was determined in the same extracts by the method of Berenblum & Chain (1938). Total N-acetylneuraminic acid (i.e. hydrolysable by 0.1 N-H₂SO₄ in 1 hr at 80°C) was determined by the thio-barbituric method of Warren (1959).

Results of all experiments were assessed for significance using Student's *t* test (Snedecor, 1948).

Results

Distribution of calcium, magnesium, sodium and potassium

Values for the distribution of calcium, magnesium, sodium and potassium in bull spermatozoa and seminal plasma are presented in Table 1. The concentration of calcium and of sodium was significantly lower in spermatozoa than in seminal plasma, whilst the reverse was true for magnesium and potassium.

Table 1. The distribution of calcium, magnesium, sodium and potassium in normal bovine spermatozoa and seminal plasma

	Calcium	Magnesium	Sodium	Potassium
Spermatozoa	23.1 ± 1.16	12.1 ± 0.54	129.2 ± 9.37	172.4 ± 9.50
Seminal plasma	35.4 ± 1.24**	9.4 ± 0.20**	253.1 ± 8.71**	133.0 ± 12.49**

The analyses were performed in aqueous suspensions of spermatozoa (5–15 mg wet wt) and seminal plasma (10–50 µl) obtained by centrifugation from sixteen samples of semen. The results (mean ± S.E.M.) are expressed in mg/100 g wet wt for spermatozoa and mg/100 ml for seminal plasma.

***P* < 0.01 (significantly different from spermatozoa).

Calcium fractions

Three fractions of calcium, namely ionized (free), complexed and protein-bound, were determined in the spermatozoa and seminal plasma, in eight samples of bull semen. Spermatozoa, in suspension in 140 mM-NaCl, and seminal plasma were subjected to ultrafiltration at room temperature for 24 hr and the total and ionized calcium were measured in the ultrafiltrates (Table 2). Although the concentration of protein-bound calcium was approximately the same in spermatozoa and seminal plasma, the concentration of ionized calcium was higher and the concentration of complexed calcium was much lower in spermatozoa than in seminal plasma.

Table 2. Calcium fractions in bovine spermatozoa and seminal plasma

Calcium	Spermatozoa		Seminal plasma	
	mg/100 g wet wt	% of total	mg/100 ml	% of total
(1) Total (before ultrafiltration)	21.9 ± 1.37	100.0	37.8 ± 1.33**	100.0
(2) Ultrafiltrable (total in ultrafiltrates)	17.4 ± 1.89	79.4 ± 2.21	31.9 ± 1.99**	84.4 ± 2.38
(3) Ionized	7.7 ± 1.23	35.1 ± 5.21	3.9 ± 0.36*	10.3 ± 0.72**
Complexed (2 – 3)	9.7 ± 1.27	44.3 ± 4.62	28.0 ± 0.30**	74.1 ± 2.11**
Protein-bound (1 – 2)	4.5 ± 0.62	20.6 ± 2.21	5.9 ± 0.72	15.6 ± 2.38

Mean values ± S.E.M. are given for eight replicates.

P* < 0.05; *P* < 0.01 (significantly different from spermatozoa).

Effect of cold shock

Cation distribution. Whole semen was cold-shocked to 0°C. The concentration of calcium, magnesium, sodium and potassium was determined in cold-shocked spermatozoa and compared with that in control samples kept at room temperature (25°C). The results, presented in Table 3, show that cold-shocking of semen caused a rise in the calcium and sodium concentration and a decrease in the magnesium and potassium concentration of the spermatozoa.

Table 3. The effect of cold shock on the concentration of calcium, magnesium, sodium and potassium in bovine spermatozoa

Spermatozoa	Calcium	Magnesium	Sodium	Potassium
Normal	23.1 ± 1.06	11.8 ± 0.52	140.7 ± 10.16	155.4 ± 9.59
Cold-shocked	38.6 ± 1.44**	8.1 ± 0.22**	186.2 ± 9.30**	101.6 ± 6.84**

Mean values ± S.E.M. are given for nineteen replicates. The analyses were performed on spermatozoa (5–15 mg wet wt) separated by centrifugation from either normal or cold-shocked samples. The results are expressed in mg/100 g wet wt.

** $P < 0.01$ (significantly different from normal).

Rate of cooling. The effect of the rate of cooling on the response of spermatozoa to cold shock was investigated in more detail. The concentration of calcium in spermatozoa in five aliquots of the same semen sample was determined after exposure to the treatments listed in Table 4. Rapid cooling of semen to 5° or 0°C caused an increase in the calcium concentration, but the increase was higher when semen was rapidly cooled to 0° than to 5°C. Cooling of semen at a slow rate had no effect.

Table 4. The effect of cooling rate on the concentration of calcium in bull spermatozoa

Treatment of semen before separation of spermatozoa	Calcium (mg/100 g wet wt)
Untreated (control)	25.1 ± 2.60
Cooled slowly from 25° to 5°C	24.9 ± 2.74
Cold-shocked by sudden cooling from 25° to 5°C	32.0 ± 2.30**
Cooled slowly from 25° to 5°C and subsequently cold-shocked by rapid cooling from 5° to 0°C	29.0 ± 3.95
Cold-shocked by sudden cooling from 25° to 0°C	40.5 ± 3.99**

Mean values ± S.E.M. are given for five replicates.

** $P < 0.01$ (significantly different from control).

Immobilization. Spermatozoa were immobilized by the two methods described in 'Materials and Methods'. Irrespective of whether the spermatozoa were motile or rendered immotile by heating just before cold-shocking, the calcium content rose, as a result of cold shock, to an approximately same level (Table 5A), but when the spermatozoa were immobilized reversibly by formaldehyde treatment, the calcium content remained stable. However, on subsequent cold-shocking it increased significantly, though not to the same extent as in formaldehyde-free spermatozoa (Table 5B).

Sonication and freezing. Five bull ejaculates were used; each was split into six samples, and these were subjected to the treatments listed in Table 5C. Homogenization (sonication) produced an increase of the calcium concentration in spermatozoa which was similar to that due to cold shock. The calcium level of homogenized spermatozoa was further raised, but the difference was not significant. When spermatozoa were frozen and thawed three times and resuspended in fresh seminal plasma, the calcium content was only slightly higher than in the untreated control ($P < 0.05$). Cold-shocking of spermatozoa which were previously frozen did not alter the calcium level,

Table 5. The effect of cold shock on the calcium concentration of bovine spermatozoa treated in various ways before separation from seminal plasma

Treatment	Calcium (mg/100 g wet wt)	Significance
A. Untreated (control)	20.2 ± 1.30	} <i>P</i> < 0.01
Cold-shocked to 0°C	39.5 ± 3.10	
Heated (10 min at 50°C)	20.5 ± 2.60	
Heated (10 min at 50°C) and then cold-shocked to 0°C	34.7 ± 4.80	
B. Diluted with Ringer-phosphate, pH 7.4		
Control	13.7 ± 0.97	} <i>P</i> < 0.01
Cold-shocked	30.2 ± 3.18	
Diluted with Ringer-phosphate-formaldehyde, pH 7.4		
Control	12.1 ± 0.64	} <i>P</i> < 0.01
Cold-shocked	18.3 ± 1.27	
C. Untreated		
Control	20.1 ± 1.34	} <i>P</i> < 0.01
Cold-shocked	39.8 ± 3.20	
Homogenized†		
Control	34.7 ± 1.86	<i>P</i> < 0.01*
Cold-shocked	36.2 ± 2.18	<i>P</i> < 0.01*
Frozen-thawed‡		
Control	23.8 ± 1.54	<i>P</i> < 0.05*
Cold-shocked	24.6 ± 2.04	<i>P</i> < 0.05*

Values are means ± S.E.M. for four (A), eight (B) and five (C) replicates.

* Compared with the untreated control in C.

† Semen was homogenized by means of ultrasonic vibration for 15 sec, as described by Harrison (1974).

‡ Spermatozoa, after separation from seminal plasma by centrifugation, were subjected to three cycles of freezing (in liquid nitrogen) and thawing and subsequently resuspended in fresh seminal plasma to the original volume of semen.

Table 6. Effect of cold shock on the distribution of calcium between ionic, complexed and protein-bound fractions in spermatozoa and seminal plasma

Form of calcium	In spermatozoa		In seminal plasma	
	mg/100 g wet wt	% of total	mg/100 ml	% of total
Total†				
Control	21.6 ± 1.54	100.00	37.8 ± 0.50	100.00
Cold-shocked	40.4 ± 2.58**	100.00	34.8 ± 0.57**	100.00
Ultrafiltrable‡				
Control	17.0 ± 1.28	78.9 ± 1.17	32.0 ± 0.82	84.7 ± 1.11
Cold-shocked	31.2 ± 2.35**	77.0 ± 1.76	29.1 ± 0.72*	83.7 ± 1.11
Ionized				
Control	6.9 ± 1.12	32.5 ± 4.88	3.8 ± 0.15	10.1 ± 0.35
Cold-shocked	8.8 ± 1.42	22.0 ± 3.17	3.2 ± 0.14*	9.2 ± 0.41
Complexed				
Control	10.1 ± 1.28	46.4 ± 4.18	28.2 ± 0.76	73.6 ± 0.98
Cold-shocked	22.4 ± 2.10**	55.0 ± 2.90	25.9 ± 0.72	74.5 ± 1.23
Protein-bound				
Control	4.6 ± 0.56	21.1 ± 1.77	5.8 ± 0.34	15.3 ± 1.10
Cold-shocked	9.2 ± 0.87**	23.0 ± 1.76	5.7 ± 0.37	16.3 ± 1.11

Mean values ± S.E.M. are given for ten replicates. The analyses of total and ionized calcium were performed in the ultrafiltrates of seminal plasma and suspensions of spermatozoa in 140 mM-NaCl prepared from normal and cold-shocked semen.

* *P* < 0.05; ** *P* < 0.01 (significantly different from control).

† Before ultrafiltration.

‡ Total in ultrafiltrates.

Ionic (free), complexed and protein-bound calcium fractions. Samples of seminal plasma and suspensions of spermatozoa in 140 mM-NaCl were prepared from normal and cold-shocked bull semen, and aliquots taken for ultrafiltration. In seminal plasma, the concentration of total ultrafiltrable calcium became lower after the semen had been cold-shocked, but the protein-bound calcium remained unchanged (Table 6). The concentration of ionized calcium in the seminal plasma was lower after cold-shocking. In spermatozoa, the concentration of ultrafiltrable and protein-bound calcium became higher after the semen had been cold-shocked, but the ratio between these two fractions remained unchanged. Cold shock produced an increase in the concentration of ionized and complexed calcium in spermatozoa, but only the increase of complexed calcium was statistically significant.

A further experiment was performed with semen which was treated with either EDTA or detergents before cold-shocking. Such treatment almost completely prevented the increase in the total calcium content in cold-shocked spermatozoa (Table 7).

Table 7. Calcium concentration in spermatozoa from bull semen that had been cold-shocked in the presence of EDTA or detergents

Treatment of semen	Additives	Calcium (mg/100 g wet wt)
Untreated	None (control)	15.6 ± 2.65
Cold-shocked	None	38.8 ± 8.20**
	20 mM-EDTA	16.3 ± 3.07
	4 mM-CTAB	15.6 ± 0.35
	6 mM-SDS	18.6 ± 0.65
	0.2% Triton X-100	20.0 ± 2.20

Mean values ± S.E.M. are given for four replicates. Semen samples were diluted with 1/10 volume of solutions of ethylenediaminetetra-acetic acid (EDTA), cetyltrimethylammonium bromide (CTAB), sodium dodecylsulphate (SDS), or Triton X-100 to give final concentration of 20 mM, 4 mM, 6 mM and 0.2%, respectively, and were subsequently cold-shocked.

** $P < 0.01$ (significantly different from control).

Interrelationships between calcium, citric acid, acid-soluble phosphorus and sialic acid in normal and cold-shocked spermatozoa

Five experiments were done to explore the nature of the low-molecular substances that might be responsible for complexing with ionized calcium in cold-shocked spermatozoa. The concentrations of calcium, citric acid, acid-soluble phosphorus and sialic acid were determined in spermatozoa separated by centrifugation from fresh and cold-shocked semen (Table 8). Only the correlation coefficient between calcium and citric acid was statistically significant ($r=0.88$; $P<0.01$).

Table 8. Effect of cold shock on the concentration (mg/100 g wet wt) of calcium citric acid, acid-soluble phosphorus and sialic acid in bull spermatozoa

	Calcium	Citric acid	Acid-soluble phosphorus	Sialic acid
Control	18.3 ± 0.75	72.0 ± 12.01	40.3 ± 4.77	199.3 ± 27.47
Cold-shocked	39.8 ± 3.83**	185.1 ± 9.40**	27.1 ± 5.01*	214.0 ± 33.54

Mean values ± S.E.M. are given for five replicates.

* $P < 0.05$; ** $P < 0.01$ (significantly different from control).

Discussion

The results of the present study broadly agree with those reported by Cragle *et al.* (1958) and Quinn *et al.* (1965) for the average values for the total calcium, magnesium, sodium and potassium levels in bovine spermatozoa, and confirm that the concentration of total calcium and sodium is lower in spermatozoa than in seminal plasma, whilst the reverse is true for magnesium and potassium.

A fact that was brought clearly to light by the present study is that, as in blood and other body fluids, calcium exists in bovine semen in at least three forms, i.e. partly free (ionic), partly complexed with small-molecular wt acidic compounds, and partly in a protein-bound form. In spermatozoa calcium is distributed fairly evenly between all three forms; the ratio between them probably depends on the concentration as well as complexing and binding strength of various substances which interact with calcium. With reference to the protein-bound calcium of spermatozoa, Brooks & Siegel (1973) have identified a calcium-binding phosphoprotein as the main acidic protein in bovine spermatozoa. The high level of calcium in seminal plasma is due largely to complexed calcium, much of it associated with citric acid.

The irreversible loss of motility and metabolic activity which the spermatozoa suffer as a result of sudden cooling is well known to be associated with increased permeability of the sperm cell, which leads on the one hand to a leakage of many intracellular constituents, including some enzymes and coenzymes, and, on the other hand, to an increased rate of uptake of certain extraneous substances, including so-called live-dead stains (Milavanov, 1934; Lasley *et al.*, 1942; Hancock, 1951; Mann & Lutwak-Mann, 1955; Mayer, 1955; Blackshaw & Salisbury, 1957; Mann, 1964; Tash & Mann, 1973).

Cold shock leads also to a serious upset in the distribution of several major cations in spermatozoa and seminal plasma, and causes in particular, a marked influx of calcium and sodium into spermatozoa, and a simultaneous efflux of potassium and magnesium; this was shown by Quinn & White (1966) and it has been confirmed in the present study. The increase in the total calcium concentration of spermatozoa, which occurs during sudden, but not slow, cooling of semen to 0° or 5°C, is characteristically reflected in a markedly elevated level of complexed and protein-bound calcium. The shift of calcium from seminal plasma to spermatozoa in cold-shocked semen occurs irrespective of whether the spermatozoa had been initially motile or not. Of special interest in this connection was the observation that when the spermatozoa are first immobilized irreversibly, i.e. by heating, then on sudden cooling they take up large quantities of calcium. But, if they are immobilized by formaldehyde according to the method of Dott & Foster (1975), i.e. in such a way that motility is arrested but not lost irreversibly, then the calcium uptake is small on subsequent cold-shocking. The formaldehyde treatment appears to render the spermatozoa less susceptible to cold shock, possibly by preventing the sperm membrane from increasing its permeability. This and other findings reported in this study, including in particular the observations on spermatozoa disrupted by sonication, lead to the conclusion that the changes which occur in the distribution and the chemical form of calcium in cold-shocked spermatozoa must be caused by damage to cell membranes. Whether or not the external lipoprotein layer of the sperm cell is directly involved in the uptake of calcium by cold-shocked spermatozoa remains uncertain. This possibility merits serious consideration in the light of some earlier but as yet unconfirmed observations by Beljkevič *et al.* (1959), and in view of the present finding that it is possible to prevent the uptake of calcium by spermatozoa by treating the semen with a surface-active agent before cold shock. This might be due to the removal by such agent of some lipoprotein from the surface of spermatozoa.

Another question of special interest is what happens to the ionized calcium of seminal plasma at the stage when it is taken up by cold-shocked spermatozoa. The results of the present study indicate that at least some of the ionic calcium becomes protein-bound. This again is probably due to changes induced by cold shock in the sperm membranes, possibly analogous in nature to the changes which Long & Mouat (1971, 1973) observed in blood corpuscles and the so-called 'ghost'-cell membranes of erythrocytes.

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